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CONTRACT NO: DAMD17-91-C-1079

TITLE: MOLECULAR ANALYSIS OF MEDAKA TUMORS: NEW MODELS FOR CARCINOGENICITY TESTING

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REPORT DATE: July 7, 1993

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Development Command, Fort Detrick
Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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Rebecca J. Van Benson — 7 July 1993

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	7 July 1993	Annual Report (10 June 92- 9 June 93)

4. TITLE AND SUBTITLE
Molecular Analysis of Medaka Tumors:
New Models for Carcinogenicity Testing

5. FUNDING NUMBERS
Contract Number:
DAMD17-91-C-1079

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER
N/A

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Development Command
Fort Detrick
Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
N/A

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release;
Distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The broad, long-term objective of our laboratory is to use fish as a model system to better understand factors which promote tumor production and to develop a reliable and sensitive means of detecting carcinogens in aqueous environments. Studies of oncogene and suppressor gene function and their relationship to tumor production in fish will contribute to our understanding of the molecular basis of carcinogenesis. In these studies we have examined the role of these genes in tumors in Japanese medaka (*Oryzias latipes*) induced by specific carcinogens. The medaka has been widely used for carcinogenicity testing in the past decade. In studies described here, we used fish which were exposed to diethylnitrosamine (DEN), and methylazoxymethanol acetate (MAMAc). A significant proportion of exposed animals developed a variety of liver tumors. In contrast, fish exposed to MNNG developed extra-hepatic tumors. DNA extracted from these tumors was analyzed in transfection assays for the presence of transforming genes. We have also initiated studies on oncogene expression in normal tissue, a study of the p53 gene and are continuing the analysis of the sequence of a novel oncogene detected in a DEN- induced cholangiocarcinoma.

14. SUBJECT TERMS	15. NUMBER OF PAGES
chemical carcinogenesis, oncogenes, suppressor genes, aquatic models, aquatic toxicology	40
	16. PRICE CODE N/A

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
unclassified	unclassified	unclassified	UL

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INTRODUCTION

For over twenty years, tumors in fish and other lower vertebrates and invertebrates have been systematically documented by the Registry of Tumors in Lower Animals at the Smithsonian Institution in Washington DC. While an overwhelming amount of data has been accumulated which documents the high incidence of neoplasia in fish residing in areas high in anthropogenic pollution and numerous studies have reported the chemical induction of tumors in fish, very little is known about the molecular basis of carcinogenesis in these animals. It wasn't until 1986 that the first oncogenes from fish were cloned and sequenced from the goldfish (Nemoto *et al.* 1986) and the rainbow trout (Van Beneden *et al.* 1986). Since that time, however, interest in fish as model systems for molecular studies has risen exponentially.

The development of aquatic models for studies in molecular carcinogenesis and toxicological studies has focused primarily on the teleosts (bony fishes). This group is the largest and most diverse class of vertebrates, with over 20,000 described species. Their phylogenetic position relative to the other vertebrates makes them ideal for comparative carcinogenesis studies. One of the best developed laboratory models is the Japanese medaka (*Oryzias latipes*). The medaka has been used for carcinogenicity testing for well over a decade (Hoover, 1984). It is well suited for these experimental studies and offers numerous advantages over rodent models: they are highly sensitive to a variety of chemicals, tumors may be induced within a short period of time and their small size allows exposure of large, statistically relevant numbers of animals at considerably less expense than rodent studies. Medaka can easily be induced to breed year round, provide large numbers of eggs and are easy to culture. The genetics, developmental biology and embryology are well-documented (Yamamoto 1975). Induction of tumors has been reported in nearly every organ by agents known to be carcinogenic to humans. Neoplastic lesions have been induced in the liver (Ishikawa *et al.* 1975; Aoki and Matsudaira 1977, Hawkins *et al.* 1988a,b, Van Beneden *et al.* in press, a,b), gills (Brittelli *et al.* 1985), eye (Hawkins *et al.* 1986), striated muscle (Van Beneden *et al.* in press,a) and in the epidermis as melanomas (Hyodo-Taguchi and Matsudaira 1984). In addition to these advantages, the medaka model offers a unique system with which to specifically address the affects of *aquatic* toxicants which are being discovered at an alarming rate in the natural environment.

In spite of the vast amount of background material available on carcinogenicity testing in the medaka (Hoover, 1984), relatively few studies have addressed the molecular basis of tumor induction and progression. It has been well established that tumor development is a multistage process of initiation, promotion and progression (Pitot, 1990). Specific classes of genes - the oncogenes and the tumor suppressor genes - are believed to play key roles in the regulation of this process. We are addressing the role of these genes in the development of tumors in medaka exposed to known carcinogens: diethylnitrosamine (DEN), methylazoxymethanol acetate (MAMAc) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). The importance of this study lies in the integration of molecular approaches with classic studies of the effects of toxicants on environmental health. The use of fish models promises to provide important contributions to the field of cancer research.

The results of the studies reported here support this premise.

Knowledge of the molecular basis of tumor induction in the medaka provide further clues in the ongoing investigation of the role of oncogenes in the development of chemically-induced tumors. The data further indicate that the medaka would be an excellent candidate for the testing of the potentially carcinogenic effects of water-borne toxicants.

BODY

I. EXPERIMENTAL METHODS

A. Tumor Induction

Medaka were exposed to three known carcinogens in separate experiments as described below:

(1) Fourteen-day-old medaka fry were exposed to MAMAc at 10mg/liter for 2 hours. Animals were then transferred to aquaria containing clean water. Fish were sacrificed at three and six months post-exposure. Livers were excised and a portion was preserved by fixation in Bouin's solution and subsequently stained with hematoxylin and eosin for histopathological analysis. The remaining tissue was immediately frozen in liquid nitrogen and stored at -70° until DNA was extracted.

(2) Exposure of fourteen-day-old fry to DEN (200 mg/liter) and subsequent sacrifice of the fish was done using a similar procedure, described previously in detail (Van Beneden *et al.* 1990).

(3) In a third exposure test, fourteen-day old medaka fry were exposed to MNNG at 30 mg/liter for one hour. Unlike MAMAc and DEN, MNNG is a direct-acting carcinogen.

B. Transfection Analysis

A transfection assay using mouse fibroblast (NIH3T3) cells modified from Graham and van der Eb (1973), was used to identify oncogenes in fish tumors. DNA used in transfection studies was extracted by quick dounce homogenization (Van Beneden *et al.* 1988; Van Beneden *et al.* in press). Ten to twenty μgs of high molecular weight fish DNA was co-transfected with the pSV_2neo plasmid in the presence of calcium phosphate. Cells were grown in the presence of G418 (Geneticin; Gibco/BRL) for two weeks and drug resistant colonies harvested by trypsinization. The cells were pooled, and divided among three assays: (a) standard focus assay; (b) nude mouse assay; (c) colony selection assay. In some cases, the third assay was omitted.

In the standard focus assay, cells were replated and grown to confluency. Selected foci were picked, expanded and DNA isolated for further analysis. Cells from the same pool were injected into athymic mice at 1.5 x 10^6 cells/mouse with 1-2 mice injected per plate of cells (Blair *et al.* 1982). Mice were examined for tumor formation at the site of injection (usually in 6-8 weeks). In the colony-selection assay, cells were replated in a defined serum-free media (QBSF, Quality Biologicals) both in the presence and absence of a low amount (0.1%) of fetal calf serum. Transformed cells formed colonies in the absence of serum, usually within two weeks.

In order to confirm that cells picked as foci in the standard focus

assay or as colonies in QBSF selection were true transformants, we expanded these cells and grew them in soft agar (McPhearson and Montegnier, 1964). In this assay, cells are suspended in a soft agar media and examined for growth after two weeks. NIH3T3 cells which normally require a hard surface to attach are unable to grow in this media. Transformed cells will grow and form small colonies in the soft agar.

C. Cloning experiments

A genomic DNA library was previously prepared in a lambda based vector (EMBL4) and a clone hybridizing to pSV_2neo was isolated, the C-7 clone. Original attempts to characterize this clone centered on the sequence analysis and restriction mapping of the portions of the DNA which did not hybridize to the vector DNA (EMBL4) or to the co-transfectant (pSV_2neo). Recently, we have altered our approach in the identification of the C-7 clone to a multi-faceted approach. These include transfection of DNA fragments from the C-7 clone into NIH3T3 cells, continued sequence analysis of other C-7 subclones, identification of transcribed messenger RNA and finally the screening of the library to identify overlapping clones.

The C7Pst1 Band 3 , Band 4 and Cla I subclones are currently being sequenced by the Sanger dideoxy chain-termination method.

RNA analysis - NIH3T3 cells transfected with the C-7 clone were used to isolate total RNA. One milligram of total RNA was isolated and used for selection of poly A^+ mRNA. Samples were analyzed on Northern blots.

Selection of other clones - Because efforts to rescreen the genomic library have been unsuccessful, we prepared a new library. Genomic DNA was isolated from NIH 3T3 cells transfected previously with medaka tumor DNA, transfection TR14. A large scale digest was performed using 150 ug genomic DNA (0.0625 U enzyme/ 6ug DNA) and the DNA loaded onto a 10-40% Sucrose gradient. The gradient was fractionated and 350 ul samples collected. Next, a portion of the samples were analyzed on a 0.3% agarose gel and the appropriate fractions combined for dialysis. These fractions were ligated to the Lambda Fix II vector (dephosphorylated) and used to transform LE 392 and P2 392 cells. The library was titered, 1.0×10^6 total pfu, and amplified (1.7×10^{12} pfu) in the selective host, P2393 cells. This library is being screened using the co-transfectant, pSV_2neo.

D. Gene Expression Analysis

Tissues were collected from different developmental stages of medaka for the isolation of RNA in order to establish the pattern of expression of cellular oncogenes during normal development. Preliminary experiments were done using liver tissue from adult medaka.

Expression of oncogenes will be determined by reverse-transcriptase PCR (RT-PCR) RT-PCR. This should allow optimization of the analysis of the relatively small amount of tissue that we have. Northern analysis of total RNA is often confusing due to interference from the large amount of rRNA present. The yield of mRNA from total RNA for fish tissue is so low (<0.2%), that amplification of specific messages from total RNA appears to be the best method for these studies with limited amounts of tissue. Accordingly, we have prepared primers specific to conserved regions of the *myc*, *H-ras* and *K-ras*

oncogenes and the *p53* suppressor gene. The first step in the development of this technique is to make a cDNA copy of the mRNA contained within the total RNA using reverse transcriptase and appropriate primers. This was accomplished by two methods. In the first, RNA was primed using random hexamers. This will non-specifically amplify all the mRNA species present. In the second method, specific downstream primers were used to initiate cDNA synthesis along a unique mRNA. The second step, which was the same for either of the priming methods, was to amplify a unique cDNA using the specific primers that we developed for the *myc* oncogene.

In order to further examine oncogene and suppressor gene expression in the medaka, a cDNA library was prepared. RNA was isolated from normal medaka adult liver (Chirgwin *et al.*, 1975) and used to prepare a cDNA library. Due to difficulties in isolating sufficient quantities of mRNA, total RNA was used in these initial attempts to make a cDNA library. The cDNA was ligated to *EcoRI* adaptors and ligated into the *EcoRI* site of the cloning vector lambda GT10 (Stratagene).

II RESULTS

A. Tumor Induction

Histopathology of the medaka tissue was evaluated by Dr. Marilyn Wolfe at the Environmental Pathology Laboratory, Inc., in Herndon, VA. Results of the samples which were used in these studies are reported in **Tables I-II**. Medaka exposed to MAMAc or DEN developed liver tumors. In animals exposed to MNNG, the target tissues varied and included gill, eye, skin and scales. The transfection assays are done as a blind study so that results were correlated with the histopathology only after the transfection analysis was completed. The pathology has not yet been determined for the MNNG-exposed fish.

B. Transfection Studies

Efficiency of the transfection assay was measured by the number of cells which survive drug treatment (i.e. exposure to G418). Cells exposed to G418 (a neomycin analog) will grow only if they have incorporated DNA from the plasmid pSV_2neo. This plasmid contains a neomycin resistance gene and was cotransfected with the fish tumor DNA.

(1) MAMAc-exposed animals - Transfection assays were used to analyze DNA samples from medaka which had been exposed to MAMAc and sacrificed at either 3 or 6 months post-exposure. Data from the transfection studies were reported last year for TR23. The fish were so small at this time that we were unable to obtain much DNA for the assays. It was also difficult to get a representative sample for histology. We have focussed our attention on the animals sacrificed at six months which had substantially larger livers and therefore provided more DNA. Later testing protocols were modified to eliminate the early sacrifice time.

DNA was isolated from MAMAC-exposed fish at 6-months post exposure and analyzed in transfection TR24. Results of these analyses were reported previously. The histopathological identity of the livers used in these studies has now been determined and is given in **Table I**. The tumorigenicity studies in nude mice are reported in **Table III**. Of the seven livers used for DNA isolation and transfection analysis, 5 were reported to have either a

cholangiocarcinoma or a hepatocellular carcinoma. Only DNA from the hepatocellular carcinomas were able to induce significant numbers of foci in the Standard Focus Assay. Cells transfected by DNA from AA-92-85-4-3 (cholangiocarcinoma) and AA-92-85-5-4 (hepatocellular carcinoma were able to induce tumors in nude mice in 3-4 weeks (**Table III**). In summary, DNA isolated from these tumors had relatively low activity in these assays. This could have been due to the very low efficiency of transfection or alternatively, the gene(s) altered by MAMAc may not be readily detectable by this type of assay.

Since the efficiency of this transfection was much lower that expected, we repeated these studies as TR25. The transfection efficiencies reported in **Table IV** indicate that DNAs used in TR25 were transfected at high efficiencies into the NIH3T3 cells. Histopathological analysis of the tissues used in this transfection is given in **Table II**. Results from the transfection analysis and tumorigenicity studies in nude mice are reported in **Tables V and VI**, respectively. We observed a significant improvement in the results of the Standard Focus Assay where 8/8 DNAs from tumor-bearing livers (cholangiocarcinomas, hepatocellular carcinomas and one mixed type) were able to induce significant numbers of foci. Significant numbers of foci were also seen when dexamethasone was added to the assay medium (5/8 tumor DNAs). This suggests that our first hypothesis was correct - that the low numbers of foci seen in TR23 was due to the very low efficiency of DNA entering the cell. Three of the cell lines transfected with tumor DNA were able to grow in the colony selection assay in low amounts of serum (**Table V**). Unfortunately, a bacterial infection destroyed all the cells in the assay with QBSF alone. Most of the transformed cells were able to induce tumors in nude mice in less than 8 weeks post injection (**Table VI**).

Secondary transfection assays (TR26 and TR26A) have been completed using DNA isolated from these primary transfectants. Results of these transfections are reported in **Tables VII-XII**. Efficiency of transfection TR26 is reported in **Table VII**. The efficiency DNA transfer in this experiment was excellent with the exception of plates TR26-9 through 12. The majority of the cells in these plates died two days after the experiment was initiated, just one day after the cells were split. All four of these plates were transfected with the same DNA, from TR25-13-A-1. We can offer no explanation for this event. There was no sign of contamination and the precipitates appeared normal. We repeated this part of the experiment as TR26A and obtained much higher efficiency of transfection (see **Table IX**).

The results of the Standard Focus Assay and the Colony Selection Assay are reported in **Tables VIII and X**, for TR26 and TR26A, respectively. Cells transfected with DNA from two primary foci (TR25-8-1-1 and TR25-13-A-1-1) were significantly transformed in both assays. The DNA originally used in the primary transfections came from livers containing both a cholangiocarcinoma and hepatocellular carcinoma. In contrast, cells transfected with DNA from TR25-14-B-2 showed only marginal transformation in both assays. The DNA used to transfect plate TR25-14 in the primary assay was originally isolated from a hepatocellular carcinoma. Although it induced tumors in the primary assay, it appears that either the transformation was not stable in the secondary assay or that this particular focus was not a true transformant. DNA from unexposed medaka controls was negative in the Standard Focus Assay and gave some background in the colony selection assay.

Results of the nude mouse assays are given in **Tables XI and XII**. Cells

transfected with DNA from one of the cholangiocarcinoma/hepatocellular carcinomas (TR25-8-1-1) was able to induce tumors in 3 of the 4 nude mice, with the earliest onset of only 2.5 weeks. This is indicative of a highly tumorigenic cell line. Cells transfected with DNA from a homologous tumor (TR26A, **Table XII**) also induced tumors in two animals within four weeks post-injection. Unfortunately, due to an error on the part of the nude mouse facility technicians, this experiment was prematurely terminated at 5 weeks post-injection. Cells transfected with DNA originally isolated from the hepatocellular carcinoma also induced tumors in 3 of 4 mice but with a longer latency period (4-5 weeks). Cells transfected with DNA originally from the hepatocellular carcinoma also caused tumors in the nude mice but with a relatively longer latent period. We also observed some background in 2 of the 5 control animals. It is unknown what is causing this high background in control animals. This problem has also appeared in control animals in the experiments of other investigators using the Frederick Facility. Dr. Don Blair, who is collaborating on this portion of the study, is currently investigating this problem.

Southern blots were prepared using DNA digests of both primary (TR25) and secondary (TR26 and TR26A) DNA. These were probed both with oncogene sequences and with high molecular weight medaka liver DNA. Hybridization to oncogene sequences was done at low stringency (35% formamide and 37^{o}C). Since genes of the *ras* family are those most often detected by transfection assays, we hybridized Southerns first to *K-ras* (from the plasmid pHiHi3 containing the mouse *c-ras*, provided by Dr. Tom Shih). As expected, fragments hybridizing to the mouse NIH3T3 *c-ras* are apparent in Pst I digests of all transfectants. Faint bands of approximately 1.9 and 0.9 kb which correspond to the two Pst I fragments in the medaka controls are also present in the transfectant digests. These may be either the medaka *K-ras* homolog or, since the signal was so weak, cross hybridization to another ras-related medaka gene.

Hybridization to medaka probes was done at high stringency (50% formamide, 42^{o}). We were unable to establish unambiguously the presence of medaka sequences in these transfectants (data not shown). In order to improve our assay methods, different restriction digests of medaka liver DNA were run on agarose gels and transferred to nitrocellulose. This Southern was hybridized to a ^{32}-P labeled medaka probe at high stringency. We were able to distinguish distinct bands in each digest which correspond to medaka repeat regions. This should enable us to isolate and identify these repeat regions which can then be used as more specific probes to confirm the presence of medaka sequences in the transformed NIH3T3 cells.

(2) MNNG-exposed animals - DNA has been isolated from the first set of MNNG-induced tumors. Unlike animals exposed to either DEN or MAMAc, who developed primarily liver tumors, the MNNG-treated fish exhibited a wide variety of external lesions. Tumors were detected on the scales, fins, epidermis, gills and in the eyes. Due to the extremely small size of these tumors, very small amounts of DNA were isolated. A transfection analysis was begun using DNA from the larger samples and results are given in **Tables XV** and **XVI**. The DNA samples which were of insufficient amount to be analyzed by transfection were saved for future PCR analysis. DNAs isolated from primary transfectants as well as DNAs from additional tumors were introduced into NIH3T3 cells. This transfection experiment is now in progress.

(3). DEN-exposed animals - Studies using DNA isolated from DEN-

exposed medaka were initiated to confirm the results of the preliminary experiments with DEN-treated animals. Transfection assays using DNA from DEN-induced tumors are still in progress. We have focussed our attention on identifying the novel transforming gene responsible for the DEN-induced cholangiocarcinoma (see also Cloning Studies). As part of this study, we investigated whether a positive clone isolated from a genomic library made from the DEN-induced cholangiocarcinoma transformed NIH3T3 cell line had retained biological activity. DNA isolated from the EMBL4 clone C-7 was used to transfect NIH3T3 cells together with carrier calf thymus DNA. This lambda clone contains a 17 kb insert, has homology to the marker pSV_2neo and presumably contains a novel medaka transforming gene (see Cloning experiments described below). Cells transfected with the construct developed numerous foci, were positive in the Colony Selection Assay (see **Table VIII**) and induced tumors in nude mice (**Table XI**) with a short latent period. These results suggest that this clone has retained biological activity. DNA was isolated from these transformed cells and used in a secondary transfection cycle.

In order to determine which portion of this 17 kb clone contains this activity, 13 overlapping Bluescript subclones were transfected into NIH3T3 cells. We hope to be able to identify a portion of this large clone which still retains biological activity and may be more easily sequenced. The C-7 clone was digested with *Xba I*, which generates at least eleven fragments not recognized by EMBL4 or pSV_2neo. These fragments were subcloned into pBluescript and amplified. The plasmid DNA from these subclones and from the Sac 2 and Sac 3 subclones was used to transfect NIH3T3 cells. Analysis of the transfection data indicated that subclones C-7 Xba 6 (2 foci/plate), C-7 Xba 9 (5 foci/plate) and the Sac 2 subclone (2 foci/plate) possessed the ability to transform the NIH3T3 cells (See **Tables XIII-XVI)** Transfection analysis also indicated that the Sac 3 subclone had a high transforming ability (26 foci/plate). However, when the Sac 3 subclone was digested with *Eco R1* and re-subcloned, these fragments exhibited no ability to transform the cells.

B. Cloning experiments

A genomic DNA library was previously prepared in a lambda based vector (EMBL4) and a clone hybridizing to pSV_2neo was isolated, the C-7 clone. Original attempts to characterize this clone centered on the sequence analysis and restriction mapping of the portions of the DNA which did not hybridize to the vector DNA (EMBL4) or to the co-transfectant (pSV_2neo). Three large fragments were obtained; the Sac 1 fragment, the Sac 2 fragment and the Sac 3 fragment. The Sac 2 and Sac 3 fragments were an appropriate size to subclone into pBluescript (SK-). These subclones have been mapped (See Figures 1A and 1B) and a large portion of the DNA sequenced. Analysis of the nucleotide sequence revealed no significant homology to any gene family for either the Sac 2 or Sac 3 subclones. Therefore, we have altered our approach in the identification of the C-7 clone to a multi-faceted approach. These include transfection of DNA fragments from the C-7 clone into NIH 3T3 cells, continued sequence analysis of other C-7 subclones, identification of transcribed messenger RNA and finally the screening of the library to identify overlapping clones.

Sequence analysis: *Pst 1* digestion of the C-7 clone resulted in the identification of a region possessing homology to radioactively labeled medaka DNA (See Seventh Quarter Report March 9, 1993). Five fragments were isolated from this region of the agarose gel and purified using the Gene Clean II kit.

Two fragments hybridized to ^{32}P-labeled medaka DNA. These are designated C7Pst1 Band 3 and C7Pst1 Band 4. Band 3 is approximately 4,000 to 4,500 bp in length and Band 4 is slightly smaller at 3,500 to 4,000 bp in length (Figure 2).

C7Pst1 Band 3 and Band 4 have been subcloned into pBluescript SK(-). Restriction analysis of the Band 3 subclone has revealed very little information. Restriction digests of the DNA with *Acc I, Bam H1, Eco R1, Hinc II, Hind III, Sma I, Sst 1, Xba I,* and *Xho I* revealed only a single site was present which occurred in the MCS (multiple cloning site). *Eco RV* and *Kpn I* revealed internal restriction sites near the MCS resulting in the presence of a 150-200 bp 5' end fragment. Digestion of the subclone with *Bcl I, Cla I,* and *Mlu I* resulted in the identification of 2 fragments each (See Figure 3). *Bcl I* and *Mlu I* do not digest pBluescript DNA therefore at least two sites occur within the insert DNA. *Cla I* cuts pBluescript once therefore one site occurs within the insert DNA. The Cla I fragment, ~1,000 to 1,500 bp, has been isolated using the Gene Clean II kit and subcloned into pBluescript.

The C7Pst1 Band 3 , Band 4 and Cla I subclones are currently being sequenced by the Sanger dideoxy chain-termination method. Comparison of the C7Pst1 Band 3 or the Cla I sequences to the sequences of the Sac 2 and Sac 3 subclones revealed no similar nucleotide composition. We therefore assume that this DNA fragment occurs within the large Sac 1 fragment region or is centered in the Sac 2 or Sac 3 fragments.

RNA analysis: Northern blot analysis of the RNA from the transfected cells and from NIH 3T3 controls revealed a high molecular weight transcript in the total transfected RNA. However, no corresponding band was observed in the poly A^+ mRNA samples. It is possible that the RNA is not polyadenlyated or that perhaps it is broken or sheared during oligo dT column isolation.

Selection of other clones: A new genomic library prepared using DNA isolated from NIH3T3 cells transfected previously with medaka tumor DNA transfection TR14. Fifteen regions, containing multiple plaques, were removed from the primary screens and subjected to a second round of plating. From this plating, thirteen possible positive plaques were selected. These individual plaques have been picked and are currently undergoing further isolation and selection to determine whether they are true positives.

C. Gene Expression Studies

Studies were initiated to examine expression of cellular oncogenes during normal development in the medaka. Total RNA has been isolated from 14 samples of 5 developmental stages of the medaka (see **Table XVII**). Yield varied from 0.18 to 2.70 mg/gram of tissue. Quality was assessed by Northern analysis which showed the presence of the two expected ribosomal RNA (rRNA) 28 and 18s bands and good quality, high molecular weight mRNA.

Pilot studies on the RT-PCR method were done using the rainbow-trout derived *c-myc* primers. Amplified cDNAs were electrophoresed on agarose gels. Only the G6PDH positive control was visible. The gels were then transferred to nitrocellulose membranes and the resulting Southern blot hybridized to a ^{32}-P labeled *myc* probe. Both the random primer and the specific downstream primer worked to amplify *c-myc* in rainbow trout liver and RTG-2 (a cell line derived from rainbow trout gonad) total RNA. Bands were not visible for the

medaka samples. Random primers worked better than the specific primer. As expected, the RNA from rainbow trout gave a weak signal, since the *myc* gene is expressed at very low levels in the normal adult liver. These results indicate that the technique is working but that the conditions need to be optimized to detect the medaka sequences. We will take two approaches to do this. First, the primer annealing temperature will be decreased to allow for less specific binding. Second, we will try primers made to different regions of the gene.

A cDNA library has been prepared from RNA isolated from over 400 normal adult livers. This will serve as the standard for comparison to genes expressed during different developmental stages. It is now being screened for the medaka p53 suppressor gene using the trout *p53* clone obtained from Dr. Soussi (Soussi *et al.*, 1990).

III. Discussion

Tumor development is a multistage process which may be subdivided into three stages: initiation, promotion and progression. Initiation is characterized by an irreversible change usually in DNA structure. Chemicals which act as initiators are also potent mutagens which react with cellular DNA either directly or after metabolic activation by the cell's biotransformation mechanisms. Although the permanent and heritable characteristics of initiated cells are well established, very little is actually known about the molecular basis of these changes. Specific mutations in proto-oncogenes have been proposed as a key step in this process. The second stage, promotion, is characterized by the reversible expansion of the initiated cell. Many chemicals which function as promoters act via receptor mechanisms, in a dose response manner. During progression, the cellular genome undergoes further irreversible alterations directly related to growth rate. (Pitot, 1990). Examination of these changes at the genetic level is just beginning.

Modern molecular oncology has focused on the interactive roles of two classes of genes involved in tumor development: the cellular oncogenes, dominant cellular genes with key roles in the control of cell growth and differentiation; and suppressor genes, recessive genes which act as negative regulators of cellular proliferation. The mechanisms of activation of cellular oncogenes include point mutations, inappropriate gene expression, chromosomal translocation and gene amplification (Bishop 1987). The functions of these genes have been extensively studied in human and other mammalian tumors, as well as *Drosophila*, *Xenopus* and yeast. Research at the molecular level in teleost fish, however, has lagged far behind. It wasn't until 1986 that the first oncogenes from fish, *ras* (Nemoto *et al.*, 1986) and *myc* (Van Beneden *et al.*, 1986a), were cloned and sequenced. Since that time the field of teleost oncogene research has exploded with efforts concentrated on the roles of these genes in the tumor formation (Van Beneden, in press). The use of fish models promises to provide important contributions to the field of cancer research.

The direct activation of oncogenes by chemical carcinogens was first described in detail in rodent models (Barbacid 1987; Sukumar, 1990). One of the best-defined systems is the activation of H-*ras*-1 in nitroso-methylurea (NMU)-induced mammary carcinoma in Buf/N rats (Sukumar *et al.* 1983) which is characterized by a specific G -> A transition of the second

base of the 12th codon. NMU is known to specifically induce such mutations by methylation of the O^6 position of deoxyguanosine. The specificity of this response was supported by later studies (Zarbl *et al.* 1985) which showed that mammary carcinomas induced by dimethylbenz(a)-anthracene (DMBA) do not exhibit the same G -> A transition. DMBA, unlike NMU, forms large adducts with deoxyguanosine and deoxyadenosine that lead usually to excision repair and non-specific point mutations. In the DMBA-induced tumors in which the H-*ras*-1 locus was activated (23%), the mutations were localized to the two deoxyadenosine residues of codon 61. The repeated detection of activated oncogenes such as *ras* in animal tumors induced by specific chemical carcinogens has important implications on the biological significance of oncogene activation in human cancers. The reproducible detection of specific transforming genes in animal model systems strongly suggests that these oncogenes have a significant role in development of certain tumors and that the mechanisms of carcinogenesis show remarkable conservation throughout phylogenetically distant models. This conservation at the molecular level validates the use of animal systems as models for carcinogenesis.

A. Transfection Analysis

MAMAc is the stable aqueous form of methylazoxymethanol (MAM), the active carcinogenic component of the naturally occurring glucoside carcinogen cycasin. MAMAc appears to be metabolically activated in tissues by esterases and NAD-dependent dehydrogenases (Grab *et al.*, 1977). The carcinogenicity of MAMAc in higher animals is well documented (Zedeck *et al.*, 1977; Sieber *et al.*, 1980). MAMAc has also been reported in previous studies to induce tumors in fish (Aoki and Matsudaira, 1981; Hawkins *et al.*, 1986; Fournie *et al.*, 1987; Van Beneden *et al.*, 1990).

The identification of the transforming gene detected in the MAMAc-induced tumors is still unknown. In order to confirm that the transformation of NIH3T3 cells is due to fish sequences, restriction digests of DNA isolated from transfected cells will be analyzed on Southern blots for the presence of fish-specific sequences. Duplicate Southern blots will also be hybridized to radiolabelled probes of known oncogenes in order to identify activated oncogenes. These studies are in progress.

Unlike MAMAc and DEN, MNNG is a direct acting carcinogen. The target organ of this carcinogen also varies in this fish. It appears to affect a number of organs, including gills, skin, eyes and scales. Molecular analysis of these MNNG-induced tumors is still in progress.

DEN is one of the most potent and extensively studied mammalian liver carcinogens. Metabolic activation of DEN via a-hydroxylation results in an electrophilic metabolite which is able to ethylate a variety of sites in DNA. In a recent study (Stowers *et al.*, 1988), DNAs isolated from DEN-induced tumors in $B6C3F_1$ mice and Fisher 344 rats were examined for the presence of activated cellular oncogenes using a transfection technique similar to the one described here. Somewhat unexpectedly, the incidence of activated *ras* oncogenes detected (14/33) in B6C3F1 mouse liver tumors was significantly lower than reported for other chemically-induced mouse liver tumors. The authors suggested that it is probable that multiple pathways exist for the formation of liver tumors in this strain of mouse. Activation of the H-*ras* oncogene may be one event in some but not all of these pathways. In contrast,

DNA isolated from only one of the Fisher 344 rats was able to produce foci in NIH3T3 cells. These results were supported by data from previous studies which reported that *ras* activation was not consistently observed in tumors in Fisher rats induced by a variety of chemicals.

DEN has been used to induce a variety of tumors, also primarily of hepatic origin, in several species of fishes (Park and Kim, 1984; Schultz and Schutlz, 1988; Grizzle and Thiyagarajah, 1988; Lee *et al.*, 1989; McCarthy *et al.*, 1991). Activated *ras* oncogenes have been detected by transfection analysis of DNA from several fish tumors. Other studies of molecular analysis of DEN-induced tumors in fish have not been reported. The gene detected in the DEN-induced cholangiocarcinoma does not appear to be homologous by Southern blot analysis to any of the known oncogenes that were used as probes. Sequence data to date support this conclusion. This strongly suggests that it may be a novel oncogene. This supports the conclusions of Stowers *et al.* (1988) of the existence of multiple pathways which do not involve the activation of *ras* genes.

B. Gene Expression Studies

The genomes of higher organisms contain approximately 100,000 different genes, only a small fraction of which are expressed in each cell. The coordinated regulation of gene expression affects all processes of cell growth and differentiation. Pathological alterations in expression are the basis for many types of cancer. In the context of this study, gene expression may be affected by either a mutation in the regulatory region of a cellular oncogene, mutational inactivation of a suppressor gene or other mechanisms which deregulate gene transcription. Alteration of oncogene/suppressor gene expression has been observed in all stages of chemical carcinogenesis (Pitot 1990) and is believed to play a key role in the conversion of a normal cell to a neoplastic state. The work proposed here to determine the expression of oncogenes during development will provide the basis for the comparison of alteration in gene expression during tumorigenesis.

C. Cloning Studies

At this time the identity of the transforming gene from DEN-induced cholangiocarcinoma is still unknown. However, available sequence information from the C7Pst1 Band 3 indicates a high degree of homology (>50%) to Wnt-1 proteins using the FASTA analysis program. When these sequences were extracted from Genebank and an alignment performed using either the Needleman and Wunsch algorithm or the Smith and Waterman algorithm for local homology the degree of sequence homology was decreased significantly (~35%). Further sequence analysis of the subclone is required to verify this information.

CONCLUSIONS

I. Significance of completed work

Results of the transfection analysis of tumor DNA from MAMAc, MNNG and DEN-exposed fish suggest that, like mammals, fish tumors have activated transforming genes which are able to transform NIH3T3 mouse fibroblasts *in vitro*.

Previous studies of a DEN-induced cholangiocarcinoma had indicated that a novel oncogene may have been activated in this tumor. Cloning and sequence analysis of this gene has not yet revealed significant homology to known genes. These preliminary findings are in support of our hypothesis that a novel transforming gene has been activated in the cholangiocarcinoma.

Analysis of the MAMAc-exposed fish is still in progress. The transfection data indicate that DNAs isolated from both a cholangiocarcinoma and a mixed cholangiohepatocellularcarcinoma are able to transform NIH3T3 cells. Southern analysis of DNA from transformed cells suggests that the suppressor gene, *p53*, may be amplified. Further studies should indicate the molecular basis of these chemically-induced tumors.

II. Recommendations for future work

It is recommended that the work continue along the directions detailed below. In addition, we suggest that the study be expanded to include suppressor genes and exposure to aquatic carcinogens. Suggested aquatic carcinogens include trichloroethylene, polycyclic aromatic hydrocarbons, polychlorinated biphenyls or dioxin-related compounds. Future studies may include exposure to more than one carcinogen, i.e. both an initiator and a promoter.

III. Work to be performed in next reporting period

Studies will continue along the following lines: **(1)** Continue analysis of MAMAc-exposed medaka; **(2)** complete the transfection studies using DNA from MNNG- and DEN-exposed fish using both Southern analysis and PCR amplification and direct sequencing techniques; **(3)** continuation of sequence analysis of the C-7 clone; **(4)** continuation of oncogene expression studies during development. **(5)** continue to characterize the medaka p53 gene.

(1) Analysis of MAMAc-exposed medaka - In order to confirm that the transformation of NIH3T3 cells is due to fish sequences, we will examine DNA restriction digests of transfected cells on Southern blots for the presence of fish-specific sequences. We will also hybridize them to known oncogene radiolabelled probes in order to identify known activated oncogenes. We will repeat the Southerns of TR23 DNA in order to confirm the presence of a mutated *p53* gene in two of the transfectants.

(2) Transfection experiments using DEN and MNNG-exposed medaka - Analysis of MNNG-exposed fish - Primary transfection analysis will be completed and secondary transfections initiated. In order to confirm that the transformation of NIH3T3 cells is due to fish sequences, we will examine DNA restriction digests of transfected cells on Southern blots for the presence of medaka-specific sequences. We are still in the process of optimizing conditions to better accomplish this. We are attempting to isolate a medaka repeat sequence. Southern blot analysis will be supplemented by the development of the PCR-based probes. This should greatly facilitate the analysis by allowing us to amplify specific codon regions of certain oncogenes (such as *ras*) and directly sequencing that portion of the gene to determine the exact mutation.

(3) Cloning experiments - During the next year, all subclones which demonstrated transforming ability in the transfection assays will be sequenced

and mapped in relation to the whole C-7 clone. We will continue efforts to map and sequence the C-7 *Pst I* subclones. A Southern blot has been prepared containing all of our Xba subclones and our Sac 2 and Sac 3 subclones. This blot will be probed with the C7 Pst 1 Band 3 DNA fragment to determine whether it actually occurs within the Sac 1 fragment or within some other region of the DNA. In addition, screening of the new library will proceed in an effort to obtain other clones possessing overlapping sequence information.

(4) Oncogene expression - We will continue to optimize methods using reverse-transcriptase PCR to identify transcripts of oncogenes and suppressor genes in RNA isolated from different developmental stages. We have demonstrated that our method works to detect the rainbow trout *myc* transcript (from which our primer sequences were developed) as well as our positive control, G6PDH. We are now focusing on determining the correct conditions to detect the medaka sequence.

(5) Isolation of the p53 gene - The medaka homologue of the *p53* gene will be sequenced and compared to the known *p53* genes.

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Table I. Histopathology of liver samples used in TR24 (MAMAc-exposed medaka)

Transfection Plate #	DNA	Histopathology
TR24-2-3	CT[1]	-
TR24-6-9	AA-92-85-1-4	hepatocellular vaculation
TR24-10-12	AA-92-85-1-17	moderate cystic degeneration
TR24-13-16	AA-92-85-4-3	cholangiocarcinoma, hepatocellular carcinoma
TR24-17-20	AA-92-85-4-6	cholangiocarcinoma, hepatocellular carcinoma
TR24-21-24	AA-92-85-5-3	hepatocellular carcinoma
TR24-25-28	AA-92-85-5-4	hepatocellular carcinoma
TR24-29-30	AA-92-85-5-7	hepatocellular carcinoma
TR24-31-32	L-88-308-4-4	hepatocellular carcinoma (DEN induced)

[1]Calf thymus DNA, control plate

Table II. Histopathology of liver samples used in TR25 (MAMAc-exposed medaka)

Transfection Plate #	DNA	Histopathology
TR25-2-3	CT	-
TR25-4	AA-92-85-1-4	hepatocellular vaculation
TR25-5	AA-92-85-1-17	moderate cystic degeneration slight/mild hepatocellular vaculation
TR25-6-9	AA-92-85-4-3	cholangiocarcinoma, hepatocellular carcinoma
TR25-10-13	AA-92-85-4-6	cholangiocarcinoma, hepatocellular carcinoma
TR25-14-15	AA-92-85-5-3	hepatocellular carcinoma
TR25-16-17	AA-92-85-5-4	hepatocellular carcinoma
TR25-18-19	AA-92-85-5-7	hepatocellular carcinoma
TR25-20-21	L-88-308-4-4	hepatocellular carcinoma
+TR25-22-25	TR23-14-1-SFA	cholangiocarcioma
+TR25-26-29	TR23-16-1-SFA	mixed hepatocholangiocarcinoma

+secondary transfection of AA-91-351-5-19 and AA-91-351-4-4, respectively

Table III. Summary of tumorigenicity assay in nude mice (TR24)

Plate #	DNA Source	# tumors/# mice	weeks to onset
TR24-2	Calf Thymus	2/2	(3 wks,5 days)/(4 wks,5 days)
TR24-3	Calf Thymus	0/2	-
TR24-6	AA-92-85-1-4	1/1	4 wks
TR24-7	AA-92-85-1-4	1/1	9 wks, 5 days
TR24-8	AA-92-85-1-4	1/1	9 wks, 5 days
TR24-9	AA-92-85-1-4	0/1	-
TR24-10	AA-92-85-1-17	0/1	
TR24-11	AA-92-85-1-17	0/1	-
TR24-12	AA-92-85-1-17	0/2	-
TR24-13/14	AA-92-85-4-3	0/1	-
TR24-15/16	AA-92-85-4-3	2/2	(3 wks,5 days)+(4 wks,5 days)
TR24-17/18	AA-92-85-4-6	0/1	-
TR24-19/20	AA-92-85-4-6	0/2	-
TR24-21	AA-92-85-5-3	0/2	-
TR24-22	AA-92-85-5-3	0/1	-
TR24-23/24	AA-92-85-5-3	2/2	9 wks, 5 days
TR24-25/26	AA-92-85-5-4	1/1	4 wks, 5 days
TR24-27/28	AA-92-85-5-4	1/1	9 wks, 5 days
TR24-29/30	AA-92-85-5-7	0/1	-
TR24-31/32	L-88-308-4-4	0/1	-

Experiment terminated at 10 weeks, 5 days

Table IV. Efficiency of primary transfection (TR25) of DNA from MAMAc-exposed medaka

Plate #	DNA Source	# drug-selected colonies/plate
TR25-2	Calf Thymus	-
TR25-3	Calf Thymus	135
TR25-4	AA-92-85-1-4	76
TR25-5	AA-92-85-1-17	92
TR25-6-9	AA-92-85-4-3	114
TR25-10-13	AA-92-85-4-6	288
TR25-14-15	AA-92-85-5-3	282
TR25-16-17	AA-92-85-5-4	> 161
TR25-18-19	AA-92-85-5-7	158
TR25-20-21	L-88-308-4-4	202
TR25-22-25	TR23-14-1-SFA	> 140
TR25-26-29	TR23-10-1-SFA	> 284

Values for plates 6 through 29 are averages of each set.

Table V. Summary of Transfection TR25 (MAMAc-exposed medaka)

Plate #	DNA	SFA (#foci/plate)	SFA/DEX (#foci/plate)	QBSF + 0.1% serum[1] (relative growth)
TR25-(2-3)	calf thymus	0	0	0
TR25-(4-5)	unexposed control	0.25	0	-
TR25-(6-9)	cholangiocarcinoma, hepatocellular carcinoma	0.29	2.4	-
TR25-(10-13)	cholangiocarcinoma, hepatocellular carcinoma	6.5	1.4	0
TR25-(14-15)	hepatocellular carcinoma	2.0	0	+
TR25-(16-17)	hepatocellular carcinoma	1.5	0	0
TR25-(18-19)	cholangiocarcinoma	0.7	0	0
TR25-(20-21)	hepatocellular carcinoma	1.5	0.5	0
TR25-(22-25)	cholangiocarcinoma	7	1.1	+1/2
TR25-(26-29)	mixed hepatocholangio carcinoma	9.75	2.4	++1/2

[1]Only a few plates in each group were examined. All QBSF plates were discarded early in the experiment due to bacterial contamination.

Table VI. Summary of tumorigenicity assay in nude mice (TR25) (MAMAc-exposed medaka)

Plate #	DNA Source	# tumors/# mice	weeks to onset
TR25-2-3	Calf Thymus	0/2	-
TR25-4	AA-92-85-1-4	1/1	7 wks, 3 days
TR25-5	AA-92-85-1-17	1/1	5 wks, 3 days
TR25-6	AA-92-85-4-3	0/1	-
TR25-7	AA-92-85-4-3	1/1	4 wks
TR25-8	AA-92-85-4-3	1/1	5 wks
TR25-9	AA-92-85-4-3	0/1	-
TR25-10	AA-92-85-4-6	1/1	7 wks
TR25-11	AA-92-85-4-6	1/1	7 wks
TR25-12	AA-92-85-4-6	0/1	-
TR25-13	AA-92-85-4-6	1/1	6 wks, 3 days
TR25-14	AA-92-85-5-3	1/1	7 wks, 3 days
TR25-15	AA-92-85-5-3	2/2	4 wks, 4 days + 8 wks, 3 days
TR25-16	AA-92-85-5-4	0/1	-
TR25-17	AA-92-85-5-4	2/2	7 wks, 3 days + 5 wks
TR25-18	AA-92-85-5-7	0/1	-
TR25-19	AA-92-85-5-7	1/1	7 wks, 3 days
TR25-20	L88-308-4-4	0/1	-
TR25-21	L-88-308-4-4	0/1	
TR25-22	TR23-14-1-SFA	1/1	6 wks
TR25-23	TR23-14-1-SFA	1/1	
TR25-24	TR23-14-1-SFA	1/1	7 wks, 3 days
TR25-25	TR23-14-1-SFA	1/1	4 wks
TR25-26	TR23-16-1-SFA	1/1	7 wks, 3 days
TR25-27	TR23-16-1-SFA	0/1	-
TR25-28	TR23-16-1-SFA	0/1	-
TR25-29	TR23-16-1-SFA	1/1	5 wks, 3 days

Experiment terminated at 8 weeks

TABLE VII
Efficiency of Secondary Transfection (TR26) of DNA from MAMAc-exposed Medaka

Plate #	DNA source[1]	#Drug-selected colonies/plate[2]
TR26-3	calf thymus	TMTC
TR26-4	TR25-4-1	122
TR26-5	TR25-4-1	112
TR26-6	TR25-8-1-1	TMTC
TR26-7	TR25-8-1-1	-
TR26-8	TR25-8-1-1	TMTC
TR26-9	TR25-13-A-1	7
TR26-10	TR25-13-A-1	7
TR26-11	TR25-13-A-1	5
TR26-12	TR25-13-A-1	4
TR26-13	TR25-26-1-1	>50
TR26-14	TR25-26-1-1	124
TR26-15	TR25-14-B-2	72
TR26-16	TR25-14-B-2	75
TR26-17	TR25-14-B-2	72
TR26-18	TR25-14-B-2	62
TR26-19	C-7 clone	67

[1]DNA for transfection TR25 was originally isolated from the following: TR25-4-1, unexposed control; TR25-8-1-1 and TR25-13-A-1, cholangiocarcinoma and hepatocellular carcinoma; TR25-14-B-2, hepatocellular carcinoma; C7, EMBL-4 clone.

[2]Numbers are an average of 2 plates (i.e. 4A + 4B). TMTC, Too many to count

TABLE VIII

Transfection TR26: Secondary Transfection of DNA from MAMAc-exposed Medaka

Plate #	DNA Source[1]	Standard Focus Assay (#foci/plate) DMEM	DMEM/DEX	Colony Selection Assay QBSF + 0.1% serum
TR26-2/3	calf thymus	0	0	0
TR26-4	unexposed control (TR25-4-1)	0	0	7
TR26-(6-8)	cholangiocarcinoma/ hepatocellular carcinoma (TR25-8-1-1)	>24	17	6
TR26-(9-12)[2]	cholangiocarcinoma/ hepatocellular carcinoma (TR25-13-A-1)	-	-	-
TR26-(15-18)	hepatocellular carcinoma (TR25-14-B-2)	1	1	1
TR26-19	C-7 (EMBL4 clone from DEN-induced cholangiocarcinoma)	24	14	10

[1]DNA was isolated from either expanded focus or colony cells from primary transfection TR25

[2]Almost all cells in this group died immediately after splitting. Reason unknown. This was repeated as TR26A.

TABLE IX
Efficiency of Secondary Transfection (TR26A) of DNA from MAMAc-exposed Medaka

Plate #	DNA Source	#Drug-selected colonies/plate
TR26A-2	calf thymus	58
TR26A-3	TR25-13-A-1 SFA	27
TR26A-4	(cholangiocarcinoma,	36
TR26A-5	hepatocellular	29
TR26A-6	carcinoma)	42

TABLE X
Transfection TR26A: Secondary Transfection of DNA from MAMAc-exposed Medaka

Plate #	DNA Source	Standard Focus Assay[1] DMEM	DMEM/DEX	Colony Selection[2] QBSF + 0.1% serum
TR26A-2	calf thymus	0	0	0
TR26A-3	TR25-13-A-1 SFA	5	48	-
TR26A-4	(cholangiocarcinoma,	6	0	12
TR26A-5	hepatocellular	>151	5	17
TR26A-6	carcinoma)	TMTC	TMTC	8

[1]Number of foci/plate; DMEM, Dulbecco's Modified Eagles Media; DEX, dexamethasone; TMTC, Too many to count

[2]Number of colonies/plate; QBSF, Quality Biologicals Serum-free media

Table XI Results of Nude Mouse Assay for TR26

ANIMAL INOCULATIONS

Cell Line	9/14/92	9/21/92	9/28/92	10/5/92	10/7/92	10/12/92	10/19/92	10/26/92	11/2/92	11/9/92	11/16/92
TR26-2	-	-	-	+	+	+	+	H**			
TR26-3	-	-	-	Died							
TR26-4	-	-	-	-	-	-	+	+	+	+	H**
TR26-5-1	-	-	-	-	-	-	-	-	-	+	+
TR26-5-2	-	-	-	-	-	-	-	-	-	-	+
TR26-6	-	-	-	-	-	-	+	+	+(H,11/6)	H**	
TR26-7	-	-	-	-	-	-	-	-	-	+	+
TR26-8-1	-	-	+	+	+	+	H**				
TR26-8-2	-	-	-	+	+	+	H**				
TR26-9-10	-	-	-	-	-	-	-	-	-	-	-
TR26-13	-	-	-	-	-	-	-	+	+	+	H**
TR26-14-1	-	-	-	-	-	-	-	-	-	-	-
TR26-14-2	-	-	-	-	-	-	-	-	-	-	-
TR26-15	-	-	-	-	-	-	-	-	-	-	-
TR26-16	-	-	-	-	-	-	+	+	+(H,11/6)		
TR26-17	-	-	-	-	-	-	+	+	+	+	+
TR26-18	-	-	-	-	-	+	+	+	+(H,11/6)		
TR26-19-1	-	-	-	-	-	+	+	+	+(H,11/6)		
TR26-19-2*	-	-	-	-	-	+	+	H**			

* Injected 9/10; all others injected 9/8 with 1-3 x 10^6 cells/mouse.

**Tumor harvested.

TERMINATED 11/21/92.

Table XII

ANIMAL INOCULATIONS[1]

Cell Line	10/15/92	10/19/92	10/26/92	11/2/92	11/9/92	11/16/92	11/23/92
TR26A-2A	-	-	-	-	-	-	-
TR26A-2B	-	-	-	-	-	-	-
TR26A-3A	-	-	-	-	-	-	-
TR26A-3B	-	-	-	-	-	-	-
TR26A-4A	-	-	-	-	-	-	-
TR26A-4B	-	-	-	-	+	+	+
TR26A-5A	-	-	L*	L*	-	L*	-
TR26A-5B	-	-	-	-	-	-	-
TR26A-6A	-	-	-	-	-	-	-
TR26A-6B	-	-	-	-	-	-	-

[1]Injected 10/15/92 @ 1-3 x 10^6 cells/mouse.

*Lump observed in groin area.

THESE ANIMALS SACRIFICED BY MISTAKE 11/23/92 - TERMINATED AT 6 WEEKS.

TABLE XIII

Efficiency of Transfection (TR28) of DNA from C-7 Subclones

Plate Number	DNA Source[1]	Number of drug-selected colonies per plate[2]
TR28-2	Calf thymus	TMTC
TR28-3	Calf thymus	TMTC
TR28-4	TR10	31
TR28-5	TR10	62
TR28-6	TR14-11Ab	72
TR28-8	subclone 1	28
TR28-9	subclone 1	39
TR28-10	subclone 2	59
TR28-11	subclone 2	72
TR28-12	subclone 3	60
TR28-13	subclone 3	59
TR28-14	subclone 4	78
TR28-15	subclone 4	64
TR28-16	subclone 5	78
TR28-17	subclone 5	72
TR28-18	subclone 6	58
TR28-19	subclone 6	42
TR28-20	subclone 7	50
TR28-21	subclone 7	67
TR28-22	subclone 8	49
TR28-23	subclone 8	48
TR28-24	subclone 9	47
TR28-25	subclone 9	43
TR28-26	subclone 10	20
TR28-27	subclone 10	not counted

[1]TR10 and TR14 are the primary and secondary transfectants, respectively, of the DEN-induced cholangiocarcinoma. TR14 DNA was used to make the library from which clone C-7 was isolated. Subclones 1-10 are derived from restriction fragments of clone C-7, cloned into Bluescript plasmid. One of these is Bluescript alone included as a negative control.

[2]Average number of colonies per 2 plates (i.e. 2A + 2B). TMTC, too many to count.

TABLE XIV

Summary: TR28 transfection of subclones of Lambda clone C-7

Plate #	DNA Source	#foci/plate SFA	#foci/plate SFA/DEX
TR28-(2-3)	calf thymus	0	0
TR28-(4-5)	TR10	20	0
TR28- 6	TR14	0	0
TR28-(8-9)	Bluescript	0	0
TR28-(10-11)	C-7 Xba7	0	0
TR28-(12-13)	C-7 Xba9	5	1
TR28-(14-15)	C-7 Xba10	0	2
TR28-(16-17)	C-7 Xba6	2	0
TR28-(18-19)	C-7 Xba5	0	0
TR28-(20-21)	C-7 Xba4	0	0
TR28-(22-23)	C-7 Xba8	0	0
TR28-(24-25)	S3-4	26	4
TR28-(26-27)	S2-2	2	0

Plates 10-27 were transfected with DNA isolated
from subclones (in plasmid Bluescript) from lambda clone C-7.

TABLE XV

Summary: TR29 transfection efficiency

(MNNG-exposed medaka and C-7 subclone)

Plate #	DNA Source	# colonies
TR29-1	calf thymus (no plasmid)	0
TR29-(2-3)	calf thymus	TMTC
TR29-4	BB-92-350-5-2	[illegible]
TR29-5	BB-92-254-4-2	144
TR29-6	BB-92-254-4-3	13
TR29-7	BB-92-006-4-1	160
TR29-8	BB-92-350-4-2	208
TR29-9	BB-92-350-5-1	TMTC
TR29-(10-11)	MD liver (unexposed controls)	64
TR29-(12-13)	Bluescript	28
TR29-(14-15)	S2Pst 2	66
TR29-(16-17)	S3Xba2	68
TR29-(18-19)	S3Eco1	33
TR29-20	S3Eco2	17

Plates 4-9 contain DNA from MNNG-exposed animals all are tumors; the pathology has not yet been determined.

Plates 12-20 were transfected with DNA from C-7 plasmid subclones.

TMTC - too many to count

TABLE XVI
Summary: TR29 transfection of DNA from MNNG-exposed animals

Plate #	DNA Source	#foci/plate	
		SFA	SFA/DEX
TR29-(2-3)	calf thymus	0	0
TR29-4	BB-92-350-5-2	0	0
TR29-5	BB-92-254-4-2	0	0
TR29-6	BB-92-254-4-3	0	0
TR29-7	BB-92-006-4-1	0	0
TR29-8	BB-92-350-4-2	0	0
TR29-9	BB-92-350-5-1	0	7
TR29-(10-11)	MD liver (unexposed controls)	2	0
TR29-(12-13)	Bluescript	0	0
TR29-(14-15)	S2Pst2	5	0
TR29-(16-17)	S2Xba2	0	0
TR29-(18-19)	S3Eco1	0	0
TR29-20	S3Eco2	-	0

TABLE XVII

Isolation of RNA from developmental stages of medaka

0 day eggs		
	0.30 ug/ul	155 ug RNA/g tissue
	0.76 ug/ul	633 ug/g
	1.12 ug/ul	-
2 day eggs		
	0.88 ug/ul	138 ug/g
	0.97 ug/ul	-
	0.23 ug/ul	-
5 day eggs		
	only one sample available - not done yet	
7 day eggs		
	1.90 ug/ul	413 ug/g
1 day fry		
	0.74 ug/ul	185 ug/g
	1.80 ug/ul	-
	3.90 ug/ul	-
14 day fry		
	1.90 ug/ul	2,700 ug/g
	8.40 ug/ul	
	1.99 ug/ul	
	5.07 ug/ul	
Adult		
	Only two samples available - not done yet	

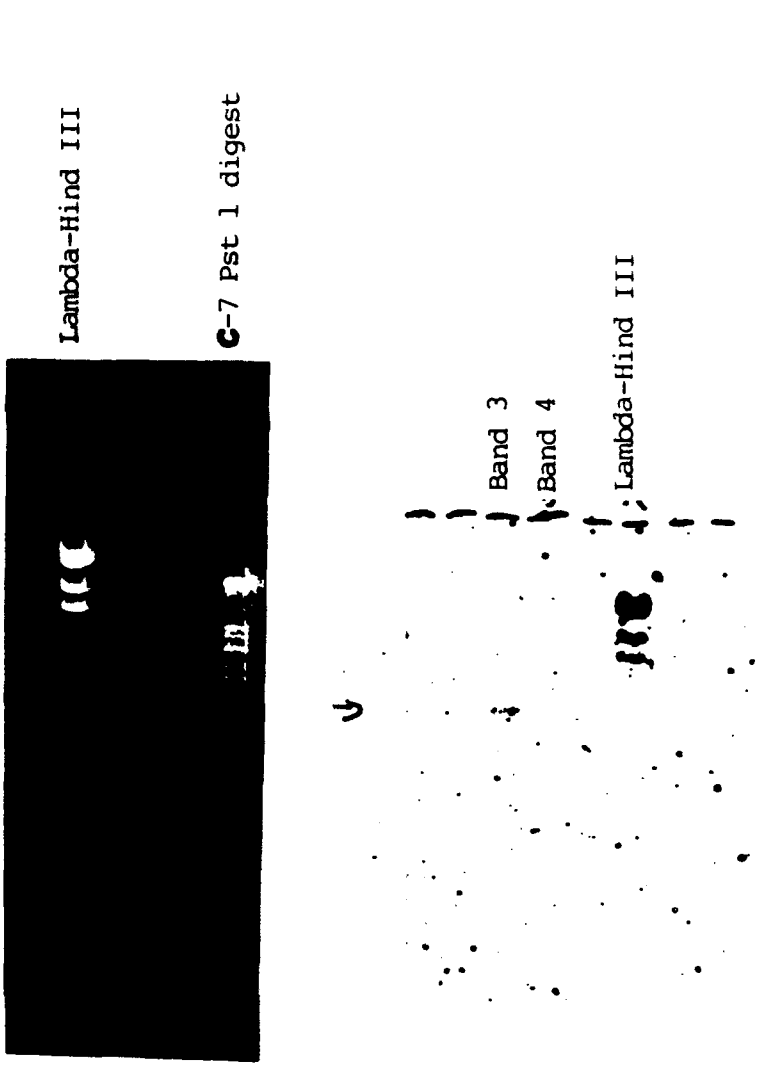

Figure 2. Restriction digest of the C-7 clone with Pst 1. Ten ug of phage DNA was digested with Pst 1 overnight at 37°C. The third and fourth fragments were isolated from the agarose gel using the Gene Clean II kit. This DNA was then fractionated by agarose gel electrophoresis and transferred to nitrocellulose. The filter was then probed with ^{32}P-labelled medaka genomic DNA.